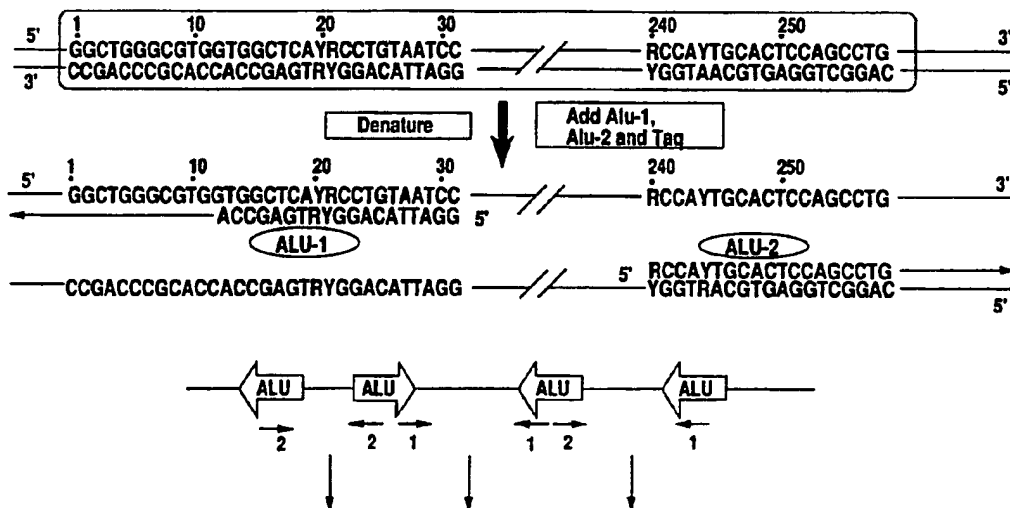




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(54) Title: IN-SITU HYBRIDIZATION PROBES FOR IDENTIFICATION AND BANDING OF SPECIFIC HUMAN CHROMOSOMES AND REGIONS

**(57) Abstract**

The invention relates to novel primer sets useful in preparing DNA probes specific for any chromosome or part of a chromosome, particularly human chromosomes. The DNA probes so produced may be used to paint individual chromosomes or portions of chromosomes in metaphase cell spreads and in interphase nuclei. When used to paint chromosomes in metaphase spreads, R-bands are readily detectable. The method is sensitive and has been shown to paint R-bands on chromosomes pieces having as few as several hundred kilobases.

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**IN-SITU HYBRIDIZATION PROBES FOR IDENTIFICATION AND
BANDING OF SPECIFIC HUMAN CHROMOSOMES AND REGIONS**

The invention relates generally to the preparation
5 of chromosome specific DNA probes that are useful in
selectively detecting individual chromosomes or
chromosome parts. The method may be used for detection
of chromosomes or parts of chromosomes in metaphase cell
spreads and in interphase nuclei by *in-situ*
10 hybridization.

Chromosome identification procedures have long been
considered an integral aspect of biomedical diagnostic
practice because of the many disease syndromes having a
15 genetic basis which are caused or diagnosed by
chromosomal alterations. Being able to observe these
chromosomal alterations in cells of the body, therefore,
not only helps in the diagnosis of the disease but has
the potential of being an effective monitor of
20 therapeutic procedures designed to eliminate the cells
with the chromosomal anomalies from the body. It is also
important to be able to rapidly monitor the chromosomes
of the fetuses of pregnancies at risk (such as in older
women, or where a parent may have been exposed to
25 environmental mutagen, or where previous siblings of the
fetus have an abnormality with known chromosomal basis).
Specific chromosomal abnormalities -- translocations
(exchanges between the arms of two different
chromosomes), inversions (internal segment of a
30 chromosome swings around and faces the opposite
direction), deletions (piece of chromosome becomes lost),
monosomies (only one instead of two chromosomes of a
type), and trisomies (three instead of two chromosomes of
a type) -- have been associated with specific sub-types
35 of cancer (1) as well as in a wide variety of inherited
or congenital abnormalities (2).

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Chromosomally based disease syndromes are often seen in hematological malignancies such as leukemia and lymphoma. The classic example is the 9:22 translocation associated with chronic myelogenous leukemia (CML).
5 Here, there is a reciprocal translocation between the end of the long arm of chromosome 9 and the middle of the long arm of chromosome 22 producing what is known as the Philadelphia (Ph) chromosome of the translocation chromosome carrying the chromosome 22 centromere (3-5).
10 The ability to see such events was made possible by a staining method developed in the early '70s which imparted to each of the 24 different human chromosomes a distinctive banding pattern (6,7). Therefore, by carrying out such a stain on cells and photographing
15 them, individual chromosomes can be cut out and lined up (according to their bands) in a procedure called karyotyping. By doing this the translocation, or any abnormal chromosome eventually stands out from the rest and is identified.

20 While effective and important in original diagnosis, this procedure is impractical for determining the percentage of such cancer cells in patients with minimal residual disease or in relapse because it is arduous and
25 labor-intensive. Furthermore, with respect to monitoring pregnancies, a faster method is preferred. A disadvantage of the procedure is its dependence on high quality metaphase spreads (structures formed when the chromosomes condense and form their distinctive
30 morphology and banding pattern during the division phase of the cell cycle). Good, scorable metaphases are often not available in the tissues which one needs to monitor (e.g., bone marrow which is the source of many leukemic cells, or amniocentesis tissues for fetus evaluation).
35 The result is that rearranged chromosomes are often not identifiable by this method leaving the entities of

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unknown origin to be referred to as "marker chromosomes".

Two groups (8,9) have described a method to stain a specific chromosome of choice. The problem associated with such a procedure was to obtain DNA from only the chromosome of interest, label it in some way so that it would be identified later (this labeled DNA is therefore chromosome-specific "probe"), and then hybridize probe to the chromosomes in a cell on a microscope slide. By visualizing the label, the chromosome was visualized since the DNA, if properly handled, hybridized only to the chromosome from which it was derived. The procedure has since come to be known as chromosome painting. Using a human chromosome 21 probe, trisomies and translocations associated with the chromosome (9,10) were visualized even in poorly defined metaphases. Specific chromosomes of interest used to prepare painting probes have been separated from other chromosomes by flow-sorting synchronized populations of dividing cells through a technically rigorous procedure requiring highly specialized and expensive equipment (a fluorescence-activated cell sorter). This leads to only a small quantity of material, so that the DNAs from the individual chromosomes then need to be extracted and cloned into cosmid libraries. To make probe, the cosmid library must be expanded, DNA extracted again and nick-translated in the presence of biotinylated nucleotide prior to hybridization to human metaphase spreads and detection of the specific chromosomes (11). An essential requirement for specific chromosome painting is the prehybridization of the probe with total human DNA in order to prevent human repeat sequences (which are not chromosome specific) from participating in the *in-situ*

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hybridization reaction.

Unfortunately, flow sorting of individual chromosomes, making and expanding and maintaining cosmid
5 libraries is problematical because of possible contamination with other chromosomes or the presence of non-chromosomal specific sequences in such material that cannot be prevented from participating in the *in-situ* hybridization. These concerns have been recently
10 highlighted (12) in studies where such probe made for human chromosome 22 hybridized to additional chromosomal regions on human metaphases. Other serious limitations of the approach include: 1) cross-hybridization of the chromosome 2 probe to the centromere of chromosome 19; 2)
15 failure of libraries made from chromosome 5 to paint chromosome 5; 3) cross-hybridization of probes made from flow-sorted chromosomes 13, 14, 15, 21, and 22 to the centromeric regions of each other; and 4) cross-hybridization of the chromosome 18 probe to the
20 centromeric regions of chromosomes 12 and 19 (as in poster presentation at the 41st Annual Meeting of the American society of Human Genetics -- 13). The method is also expensive and limited to the availability of the flow sorted libraries. The procedure is relatively
25 inflexible. For instance, there are situations in which one might be interested in painting only a portion of a specific chromosome (e.g., the p-arm of chromosome 16). This has not yet been achieved using flow-sorted libraries, yet this would be an excellent probe for the
30 identification of inversions associated with cancer and other disease syndromes. An example of this is acute nonlymphocytic leukemia where there is a pericentric inversion involving both the short and long arms of chromosome 16 (14). Probe for only the short arm of the
35 chromosome would identify the inversion chromosome as one which had staining on portions of both arms whereas a

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normal chromosome 16 would have only the short arm painted. Finally, probe made from flow sorted chromosome libraries does not allow the identification of the regions of the respective chromosomes brought together by the rearrangement because the longitudinal differentiation (banding) of the specific chromosome is lost and probes to paint specific portions of chromosomes are not available by this method. Therefore, the approach is not effective in identifying either break point sites in rearrangements or deletions (or other events in which only a single chromosome is affected).

Another way to isolate human chromosome-specific DNA is amplification (by polymerase chain reaction -- PCR) from interspecific hybrid cells containing only the human chromosome or chromosomal region of interest. Human-rodent hybrid cells monochromosomal for virtually every human chromosome and for portions of a human chromosome are now available. Since it is known (15) that the human genome has hundreds of thousands species-specific repeat sequences scattered throughout, several groups (16-19) have prepared consensus primers to bind to these sequences. By using these primers for PCR amplification, some success in pulling out human chromosome specific DNA sequences from hybrid cells and various types of recombinant DNA libraries have been achieved. However, a recently reported attempt to make painting probe from such material (20) resulted in speckled chromosomes with high background with no possibility of observing any longitudinal differentiation of the painted genetic element. Although selective in amplifying human DNA from hybrid cells, sensitivity in chromosome painting was low. Thus, the reported methods are impractical for developing probe useful for the identification of deletions or translocation breakpoints in abnormal cells.

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An attempt to paint specific human chromosomes with the total DNA from a hybrid cell containing the human chromosome of interest has met with partial success (21). Unfortunately, for most of the chromosomes attempted, no clear specific painting was obtained. In the few cases where success in painting a specific chromosome was achieved, the entire chromosome was painted and there was no possibility of observing longitudinal differentiation.

Identification of cells having chromosomal rearrangements at known breakpoints on the affected chromosomes has been accomplished by using, as probe, sets of cosmids that flank the breakpoint. Use of such cosmid probes flanking the breakpoint region of the p-arm of chromosome 16 has enabled visualization of the inversion associated with acute nonlymphocytic leukemia (22). However, the intensity of the signal was relatively weak. One would expect a much stronger signal if the entire arm of the chromosome were painted. Using a similar technique, the CML Ph chromosome has been identified using a pair of cosmids, one from the bcr gene proximal to the breakpoint on chromosome 22 and the other from the abl gene distal to the breakpoint on chromosome 9 (23). The intensity of the signal appeared weak, raising doubt that it could be reliably used to identify the breakpoint in the majority of cells without computer enhancement. These latter two approaches, while somewhat effective for chromosomal alterations where genomic regions flanking the breakpoints have been cloned, are useful only where such sites have been precisely identified or isolated. In the vast majority of cases, the sites are unknown and there is no effective method of identification.

Finally, DNA probes have been developed that specifically detect the centromeres (the dot-like

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structure at the junction of the two chromosome arms) for particular chromosomes (24). These probes are supposed to be effective in determining the number of chromosomes (for chromosomes from which such probes have been developed) in cells (24). However they not only provide no information on rearrangements involving those chromosome but often lack specificity in that probe developed for the identification of one centromere will often cross-hybridize to centromeres of other chromosomes.

Therefore, progress has been made in developing techniques to selectively identify individual human chromosomes. However, current methods have several shortcomings, including: (1) lack of detection of abnormal chromosomes in less than ideal metaphase spreads; (2) impracticality in determining the frequency of abnormal cells in a complex tissue; (3) inefficient detection of identify of marker chromosomes; (4) lack of specificity in identifying sub-chromosomal regions; (5) great time and expense involved in either karyotyping or preparing probe; (6) lack of flexibility; and (7) failure to paint chromosomes adequately while still observing landmarks for longitudinal differentiation.

The present invention is designed to address these problems. It provides a highly efficient and selective means for identifying individual chromosome abnormalities, as well as marker chromosomes in interphase as well as metaphase nuclei; has the flexibility to develop probe for specific chromosomal regions; and permits the efficient manufacture of probes that will not only brightly paint a human chromosome of interest, but will also paint on that chromosome a banding pattern that will enable the identification of the sites of chromosomal aberrations.

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The invention is a method to prepare DNA probes that are specific for virtually any desired chromosome or part of a chromosome. A novel aspect of the probes is that they are relatively free of repeat sequences so that a probe directed to a particular chromosome or part of a chromosome will hybridize only with that chromosome or part of a chromosome. The probe will not hybridize to other chromosomes of the same species because the probe will have essentially only unique DNA sequences of the desired chromosome.

In general, chromosome specific DNA probe may be prepared by first isolating the desired DNA and then amplifying only regions of chromosome specific DNA. This is achieved by hybridizing primers to certain regions of a species-specific repeat sequence, then amplifying. The DNA between the repeat segments is amplified, but little if any of the repeat segment is synthesized. The resulting DNA probe is essentially free of species specific repeat DNA sequences to which the primer was hybridized.

In the practice of the invention, one should determine or select the particular a chromosome or part to which probes are desired. The chromosome may be selected from any number of sources, including insects, birds, fish, humans, animals or any living thing that has a chromosome with chromosome-specific DNA sequences and a sufficient number of interspersed segments with species specific repeat DNA sequences. Mammals, including humans, have such interspersed segments, for example, LINES or SINES. Clustered repeat sequences are also found in some species, for example the alphoids found in humans. One type of SINE found in mammalian species is the Alu repeat sequence. The chromosome, or portion thereof, need not be free of chromosomes from other

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species as long as its species specific segments are different enough in sequence from the repeat segments of the other species so that primers for polymerase chain reaction (PCR) may be prepared which will selectively bind to the sequences of the repeat segments associated with desired species.

DNA for use in the preparation of probes may be obtained from the any source in which the chromosome, or portion thereof, has been isolated from the remainder of that species other chromosomal material. These sources include interspecific somatic cell hybrids, flow-sorted preparations, microbial vector clones, or microdissected DNA. In a most preferred embodiment the chromosome or portion thereof to which probe is being prepared is isolated in interspecific somatic cell hybrids, such as hamster.

In the practice of the invention, it is typical to prepare two primer sets for use in subsequent PCR amplification, each set having at least one primer which is capable of hybridizing to a region within a repeat segment. One or more primers from the first primer set will preferably bind at or near the 5' terminus of such repeat segments within the chromosome or chromosome fragment. The primer or primers will facilitate DNA replication in the direction of the repeat DNA segment 5' terminus. Similarly, one or more primers from the second set of primers will preferably bind at or near the 3' terminus of a repeat segment and will facilitate DNA replication in the direction of the 3' terminus.

After the primers are prepared, an annealing step is performed by hybridizing with DNA obtained from the material containing the chromosomal region of interest. Annealing by hybridization techniques is well known to

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those skilled in the art and generally is conducted at a temperature between about 50°C and 60°C. In an important aspect of the present invention, when interspecific somatic cell hybrids are the source of the chromosomal material for which probe is being made, hybridization is performed at a higher temperature, in the range of 60°-70°C, most preferably at 65°C. This prevents annealing of the primers to repeat segments in chromosomes of the other species.

10

A final step in preparing chromosome specific DNA probes is amplification of the inter-repeat regions of the selected chromosome or chromosome part. For human chromosomes, the DNA amplified will be specific for that particular chromosome or part. Since DNA synthesis is directed away from the middle and off the termini of human Alu repeat segments, the DNA probe so produced will contain a minimum amount of Alu repeat sequences. A small amount of Alu repeat sequence amplified may arise because the primer sets do not bind to the very ends of the Alu repeat segments.

20

In humans as well as other mammals, species specific repeat sequences occur frequently. Segments of Alu repeat sequences are the most prevalent middle repeat elements in human chromosomes. When the method is used to prepare human chromosome or human chromosome portion-specific DNA probes, the first primer set (Alu-1) is designed to bind to base pairs 13-31 from the 5' end of 300 bp Alu segment. In a preferred embodiment, primers in this first primer set have a sequence which is reverse complementary to the Alu consensus sequence located from base pair 13-31. This means that priming with Alu-1 will direct DNA synthesis toward the 5' terminus, and away from the middle, of the Alu segment in which the primers bind. In a most preferred embodiment, complementarity to

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the consensus sequence is sufficiently degenerate so that binding to a large number of human Alu segments occurs. This is accomplished using a set of primers synthesized so that, in combination, the primers will bind to
5 virtually all Alu segments. In this embodiment, the primers do not bind with Alu segments of nonhuman species.

In a most preferred embodiment, members of the Alu-1
10 set have the base sequence GGATTACAGGYRTGAGCCA. Y is either pyrimidine T or G and R is either purine A or G. The Alu-1 primer set most preferably comprises four primers with either thymidine or cytosine at position 11 and adenine or guanidine at position 12. In a most
15 preferred embodiment the primers are used in approximately equal amounts. These primers will bind to a large number of human Alu consensus segments but not to nonhuman Alu segments. It will be appreciated by those of skill in the art that other primers may be effective
20 in the practice of the invention, such as somewhat shorter or longer primers. Also, fewer than four primers may be used. The closer the primer binds to the 5' end of an Alu 300 bp repeat segment, the less Alu repeat sequence DNA will be obtained on subsequent amplification
25 of chromosome specific DNA. Although binding at the very end of human Alu sequences is preferred, Alu sequences are less conserved at that position and a sufficiently consensus sequence is not identified. It is preferred that the primer bind to as many Alu regions as possible
30 in order to promote amplification of as much chromosome specific DNA as possible. It should also be appreciated that repeat segments other than Alus occur in human chromosomes. Analogously designed primer sets could be used with the Alu primers to eliminate essentially all
35 repeat segments.

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A second primer set (Alu-2) has at least one primer and is also designed to bind with a human Alu consensus sequence, but these primers will preferably bind at or near a 3' terminus of a 300 bp Alu segment, not at the 5' end. The primers in Alu-2 have a base sequence identical to a base sequence within an Alu repeat segment and therefore prime to direct DNA synthesis in a 3' direction, that is, away from the center of the 300 bp Alu segment to which they are annealed. A preferred consensus sequence is located within 100 base pairs of the 3' terminus, most preferably at base pair position 240-258 of 300 bp Alu repeat segment. The primers in Alu-2 most preferably have base pair sequences that bind with the majority of human Alu repeat segments. This is accomplished by taking into account the degeneracy of consensus sequences within Alu segments and preparing a mixture of primers with base sequences which bind with nearly all the consensus sequences. The primers in the Alu-2 set comprise primers having the base sequence RCCAYTGCACTCCAGCCTC where R is A or G and Y is T or C. A most preferred number of primers in the second primer set is four and they are most preferably used in approximately equal amounts.

In a particular aspect of the invention, human chromosomes or parts are used to prepare specific probe. For example, any of the 24 different human chromosomes (number 1-22, the X and the Y) may be isolated in various hybrid cells and used to prepare probe specific to that chromosome by the described method. Additionally, a portion of a chromosome may be used, for example, the p-arm of chromosome 16, band q13.3 of chromosome 19, 1q, 3q, 5q, 7q, 9q or 22q. The method has been demonstrated with portions of a chromosome as small as 1-2 Mb of human DNA from band q13.3 of human chromosome 19. The lower limit of detection may be as low as 500 base pairs in

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situations where specific DNA bracketed by repeat sequence is obtained and amplified as probe by the method described.

5 DNA of the desired chromosome may be isolated from species total chromosomal material by flow sorting or, preferably when chromosome parts are desired, from interspecific somatic cell hybrids or artificial yeast chromosomes. Isolation may also be by microdissection of
10 the desired DNA.

The invention also relates to a method for painting a human chromosome or portion of a human chromosome in metaphase spreads or interphase nuclei. The DNA probes
15 prepared are specific for the chromosome or part selected. The probes are then labeled with a substance that can be later detected, for example, biotin, digoxigenin, ^{32}P , dinitrophenol, aminoacetylfluorene or conjugated mercury, and incubated with a sample in which
20 the human chromosome or portion is to be identified by *in-situ* hybridization, conjugated, for example, with avidin-fluorescein, Texas red, fluorescein isothiocyanate or acid phosphatase and detected by one of a series of methods such as fluorescence, autoradiography or
25 chemoluminescence. These are procedures well known to those skilled in the art. Biotin is the preferred label, avidin-fluorescein the preferred conjugant, and fluorescence the preferred detection method.

30 Small amounts of amplified repeat sequences amplified from the human chromosome of interest and which are not chromosome specific may be prevented from participating in the *in-situ* hybridization reaction. In another innovative aspect of the invention, this is
35 accomplished by blocking those non-chromosome-specific regions with middle- and highly- repetitive DNA isolated

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from the total DNA of the species, most preferably Cot 10 DNA.

The invention also includes a method of removing
5 small amounts of amplified repeat sequence DNA from the
in-situ hybridization probe using PCR amplified Alu
repeat sequence terminal regions. Plasmid comprising a
complete 300 bp species specific repeat Alu sequence is
used, preferably Blur2. Two primers are then prepared
10 that will anneal each of the two plasmid arms. These
primers, and Alu-1 or Alu-2 primers are annealed to DNA
from the plasmid. PCR is conducted in order to amplify
the repeat sequences from the two ends of the Alu segment
(bp 1 - bp 31 and bp 240 - bp 300). The amplified Alu
15 terminal regions are linked to a carrier, then the
labeled Alu sequences from the probe DNA are hybridized
to them. This removes the terminal Alu repeat sequences
from the probe to be *in-situ* hybridized. A preferred
carrier useful for linking with amplified Alu terminal
20 regions is diazobenzoxymethyl cellulose although other
carriers such as diazotized plastic beads may also be
used. Where repeat sequences are prevented from
participating in the *in-situ* hybridization reaction by
blocking, the efficiency of that blocking reaction can be
25 increased by the addition of the amplified Alu terminal
regions to the middle and highly repetitive DNA used for
such purpose.

The chromosome painting method may also be applied
30 to painting of R-banding patterns on specific human
chromosomes in metaphase spreads. Good metaphase spreads
are not critical.

The present invention also contemplates the use of
35 human Alu primer sets and specific chromosome probes in
kit form. Thus in a preferred mode of use, lyophilized

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human specific inter-Alu-1 and inter-Alu-2 primers could be used to conveniently amplify a selected human chromosome or portion of human chromosomes to produce DNA probe directed to that particular DNA. In yet another preferred mode of use, labeled DNA probe specific for selected human chromosomes would be provided to use as probe. This could include DNA probes for human chromosomes 1-22 and the x and y chromosome and also chromosome parts, such as 16p, 19 q13.3, 9q, 5q, 7q, 3q, 1q and the like.

The invention also includes two unique primer sets each comprising four primers especially designed for use in the production of human specific probe for any desired chromosome or part thereof. Each set may comprise one or more primers but is most preferably four primers. The base sequence of one primer set is GGATTACAGGYRTGAGCCA and the base sequence of the second primer set is RCCAYTGCACTCCAGCCTG where Y represents T or C and R is A or G. There are four primers in each set and in a most preferred mode of use are used as mixtures of approximately equal amounts.

Figure 1 shows the agarose gel separation of inter-Alu-PCR products using both Alu-1 and Alu-2 primers (1/2), Alu-1 primer only (1), or Alu-2 primer only (2) on HeLa DNA and CHO DNA. Unmarked channels are size markers.

Panel A. (Left). PCR conducted under standard conditions, annealing temperature 55°C.

Panel B. (Right). PCR annealing temperature at 65°C.

Figure 2 shows an agarose gel separation of inter-

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Alu-PCR products after using the Alu-1/Alu-2 primer sets (O) or a published Alu PCR primer (N) on human genomic DNAs from a series of cosmids (numbered 1-5).

5 Figure 3 is a photomicrograph of a metaphase from hybrid 5HL9-4, which contains human chromosome 19 as the only human genetic element, *in-situ* hybridized using total human DNA as probe. The arrow indicates the only fluorescing chromosome.

10

 Figure 4 is a photomicrograph of a human metaphase spread after *in-situ* hybridization with inter-Alu-PCR probe obtained from the hybrid containing human chromosome 19 as the only human genetic element. Arrows
15 indicate two fluorescing chromosomes, identified as the human 19 chromosomes. Note the absence of fluorescence at the centromeres (tips of the arrows).

 Figure 5 is a photomicrograph of a metaphase of a
20 hybrid cell (41XP91-3) containing the p-arm or human chromosome 16 as the only human genetic element following *in-situ* hybridization with total human DNA. The arrow indicates one small fluorescing fragment containing human DNA.

25

 Figure 6 is a photomicrograph of a human metaphase spread following *in-situ* hybridization with inter-Alu-PCR probe made from the hybrid (41XP91-3) containing the p-arm of human chromosome 16 as the only human genetic
30 element. Arrows indicate both p-arms of human chromosome 16 as the only fluorescing elements.

 Figure 7 is a photomicrograph of metaphase following
35 *in-situ* hybridization with total human DNA on a hybrid cell line (2F5) containing from 1 - 2 Mb of human DNA from the q-arm of human chromosome 19 as the only human

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genetic element. That element is translocated onto a CHO chromosome. A small region of fluorescence on an otherwise non-fluorescent chromosome is indicated by the arrow.

5

Figure 8 is a photomicrograph of two human metaphase cells following *in-situ* hybridization with inter-Alu-PCR probe made from hybrid 2F5. Arrows indicate the location of narrow bands of fluorescence appearing only on both chromosome 19 q-arms.

10

Figures 9 and 10 are photomicrographs of two human metaphases following *in-situ* hybridization with inter-Alu-PCR probe made from the hybrid containing only human chromosome 7. The arrow indicates the location of two chromosomes with fluorescence in each cell. The fluorescence produces an R-banding on the chromosomes.

15

Figure 11 is a photomicrograph of a human metaphase following *in-situ* hybridization with inter-Alu-PCR probe made from DNA of a hybrid containing chromosome 5 as its only human genetic element. Note the presence of only two fluorescing chromosomes in the cell with the broad R-band pattern distinctive for human chromosome 5.

20

Figures 12 and 13 are photomicrographs of human metaphases following *in-situ* hybridization with inter-Alu-PCR probe made from a hybrid containing chromosome 17 as its only human genetic element. Both chromosome 17s are lit up in both figures. Where the chromosome is more condensed (figure 12) the R-bands are closer together and the R-banding pattern is not obvious. The chromosomes are identified in poorly spread metaphases as shown in Figure 13.

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Figure 14 is a photomicrograph of a human metaphase

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following *in-situ* hybridization with inter-Alu-PCR probe made from a hybrid containing chromosome 3 as its only human genetic element. Both chromosome 3s are the only fluorescing elements in the broad R-band pattern, with a dark centromeric region characteristic of human chromosome 3.

Figure 15 is a photomicrograph of a human leukemic cell with a known chromosome 16 inversion after *in-situ* hybridization with inter-Alu-PCR probe made from a hybrid containing only the p-arm of chromosome 16. At the bottom of the field is the unaffected chromosome 16 with only the p-arm fluorescent. Note the inversion chromosome with fluorescence not only the end of the p-arm but also in the middle of the q-arm (arrow).

Figure 16 is a photomicrograph of a metaphase from a leukemic cell line with a known chromosome 7 deletion following *in-situ* hybridization with the inter-Alu-PCR probe made from a hybrid cell containing human chromosome 7 as its only human DNA content. In addition to a banded fluorescing intact chromosome 7, a second banded fluorescing chromosome 7 deleted distal to the bright band on the proximal region of the long arm is shown indicated by the arrow.

Figures 17 and 18 are photomicrographs of normal human interphase nuclei following *in-situ* hybridization with the inter-Alu-PCR probe made from hybrid 2F5 which contained only 1 - 2 Mb of human DNA from band q13.3 of human chromosome 19. Notice the bright, punctate fluorescence resolving each of the domains occupy these regions of chromosome 19 in the interphase cells.

Figure 19 is a photomicrograph of an interphase nucleus of a human leukemic cell with a known chromosome

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16 inversion after *in-situ* hybridization with inter-Alu-PCR probe made from a hybrid containing only the p-arm of chromosome 16. Two bright domains of hybridization are interrupted by non-fluorescence as indicated at the
5 arrow.

The invention relates generally to the preparation of human chromosome specific probes that are essentially either blocked for or free of non-chromosome specific
10 repeat sequences. These probes when applied to human cells via the process of *in-situ* hybridization, will identify the human chromosomal region of interest. The procedure will allow the rapid identification of chromosomal abnormalities associated with various forms
15 of cancer and birth defects.

A novel aspect of the invention is the design, preparation and conditions for use of two sets of consensus Alu-PCR primers for manufacture of the
20 chromosome specific probes by inter-Alu-PCR. The scheme takes advantage of the fact that Alu segments are distributed throughout the human genome and that by using them to instigate PCR reactions, unique, chromosome-specific sequences located between such segments may be
25 amplified for use as probe for chromosome identification. Alu sequences are of the most abundant family of middle repetitive DNA sequences in the human genome. About 500,000 Alu family members are present in the haploid human genome, amounting to about 6% of the total mass of
30 the DNA (25). These Alu sequences are not precise copies of a single sequence but instead are related copies of a consensus sequence which is approximately 300 base pairs (bps) long and consists of two monomer units.

35 Alu primers were designed based on several considerations. First, it was necessary to insure that a

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maximum number of Alu sequences were annealed by the primers. It is estimated that the average divergency of Alu sequences from the consensus is 12% (26). This divergency (or degeneracy) needs to be considered in the design of the primers in order for them to anneal with as many Alu sequences as possible. Second, since the Alu sequences themselves are not chromosome specific, it was necessary to insure minimum representation of the Alu sequence themselves in the product of the inter-Alu-PCR without impairing the first consideration. Therefore, the primers had to be designed to recognize the ends of Alu segments and direct priming away from the middle of such segments. Third, the primers needed to be designed so that inter-Alu-PCR would occur independent of the orientation of Alu segments in the human DNA to maximize the amount of unique sequence amplification. Finally, the primers needed to be human specific in order to amplify selectively human DNA in the presence of excess DNA from other species.

20

The Alu primers were designed based on a current revision of consensus sequence of human Alu repeats (25). The revision was based on nucleotide sequences of 50 different, cloned and sequenced human Alu segments. Two regions on the sequence showing high degree of conservation among Alu family members were identified. One is at bp 10-50 and the other at bp 240-260. These were the candidate regions for the primer locations. One provided the basis for design of the first primer (Alu-1) that bound near the 5' end of a 300 base pair (bp) Alu segment, and a second stretch was used to design the second primer (Alu-2) which bound near the 3' end.

In considering the first or 5' region, it was noticed that there was a lack of sequence conservation around position 20. At position 20, 31% of the segments

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had a T rather than C; and at position 21, 42.5% of the Alu sequences had an A instead of G. In order to maximize binding of primers at this region, degeneracy at these two positions was taken into consideration in primer design. Bp 10-12 could not be used because there were substitutions of more than one nucleotide at those positions. Therefore, the Alu sequence selected for the design of the Alu-1 primer went from bp 13 to bp 31 and, taking degeneracy into consideration, had the sequence TGGCTCA(C/T)(G/A)CCTGTAATCC. In order to minimize the incorporation of Alu sequence itself in the product of the inter-Alu-PCR, the primer was designed to recognize that region and to direct DNA synthesis off the 5' end and away from the middle of the Alu segments to which it bound. Therefore, the actual Alu-1 primer is the reverse complement of that sequence or GGATTACAGGYRTGAGCCA (where Y is either pyrimidine, C or T; and R is either purine, A or G).

For the second or 3' region there were regions of degeneracy at bp positions 240 and 244. At bp 240, 37.5% of the Alu members had an A instead of G. At position 244, 32.7% had a T instead of a C. For the same reason as above, degeneracy was incorporated into the primer design and the sequence of primer Alu-2 is RCCAYTGCACTCCAGCCTG. This is a direct and not an inverse complementary sequence directing priming off the 3' end of an Alu segment.

Since each primer reads away from the middle of the Alu segment, the resultant inter-Alu-PCR will not only be initiated from a maximum number of Alu segments but also incorporate a minimum amount of Alu sequence. Furthermore, the design takes into consideration the fact that two adjacent Alu sequences could be arranged head to head, tail to tail or in tandem. Having one primer at

-22-

each end with the direction of DNA synthesis away from the Alu segment enables amplification between any two Alu sequences independent of their orientation when the two Alus are within appropriate distance for PCR. This
5 yields product ranging from a few hundred to several thousand base pairs. Therefore, the primer design maximizes both the number of Alu segments recruited and the number of inter-Alu unique sequences amplified while minimizing the amount of Alu sequence incorporated into
10 the reaction products and defines the DNA probes as practiced by this invention.

Comparing Alu-1 and Alu-2 with rodent Alu-like consensus sequences (27) indicated that 11 nucleotides at
15 the 5' end of Alu-2 are not present in either Chinese hamster or mouse. For Alu-1, there are 7 mismatches in Chinese hamster sequence and there is no corresponding sequence in mouse. Therefore, it was possible to establish conditions for the specific amplification of
20 human DNA using these two primers in the presence of rodent DNA. Thus, when there is only one, or part of only one, human chromosome in the DNA from a human x rodent cell line subjected to the PCR, the amplified DNA will be specifically from that human chromosomal region.
25 This material can then be used for in-situ hybridization to identify the location of those unique sequences in the human genome.

As a second innovative feature, this method is
30 designed to be used on DNA from interspecific somatic cell hybrids that retain only a single, or part of a single, human chromosomal element isolated against a rodent chromosomal background. By conducting the PCR reaction, under conditions described below, on the DNA
35 from such cells using the primers designed above, only the human DNA (representing the human chromosomal

-23-

material on interest in the hybrid cell) will be manufactured in large quantity by PCR amplification.

5 In this procedure, the temperature conditions for the crucial annealing step of PCR are elevated above the standard temperature used for the reaction because variations of the consensus sequences recognized by the primers (Alu-1 and Alu-2) also exist (as indicated above -- 27) in the rodent versions of Alu segments (Alu sequences have some level of evolutionary conservation in mammalian species). At standard temperatures of annealing, the primers may bind to rodent Alu sequences of shared homology, thus decreasing specificity toward human chromosomal material present in the hybrid cell. 10 However, at higher temperatures, the sequences of the primers and the human Alu sequences to which they are designed to bind, must be more precisely matched allowing the Alu primers designed to recognize consensus human Alu sequences to amplify only the human chromosomal material present in the DNA. 15 20

For this amplified DNA from a specific chromosomal region to function effectively as probe to identify that specific chromosomal region in a human cell, repeat sequences present in the DNA must be prevented from taking part in the *in-situ* hybridization reaction for which the probe material will be used (8,9). This is necessary since, in addition to the chromosomal-specific unique sequences, the inter-Alu-PCR reaction will amplify 25 Alu repeat sequences located at the ends of the Alu segment sites of priming as well as other types of non-chromosomal-specific repeat sequences which might be located between Alu segments participating in the reaction. Since such repeat sequences are not specific 30 for the chromosomal region of interest, in the *in-situ* hybridization reaction, they will hybridize to 35

-24-

chromosomal regions on other human chromosomes. These repeat regions were effectively prevented from participating in the reaction by designing a blocking agent made from total human DNA. This was accomplished with high efficiency isolation of a repeat sequence-rich fraction of human DNA (called low-Cot DNA) and prehybridizing to probe DNA. This effectively blocked the participation of the repeat sequence regions in the *in-situ* hybridization reaction and gave the probe its specificity to the chromosome region of interest.

Another important unique feature of this approach is that preparing probe in this manner (inter-Alu-PCR) results in probe that not only binds specifically to the human chromosome of interest but will also impart to that human chromosome a banding pattern. This makes it possible to identify regions where the normal chromosome structure has been modified (by deletion, translocation or inversion). The observed banding pattern is resolved because Alu sequences are not homogeneously distributed throughout the human genome but rather are in higher concentration in specific regions (R-bands) along the length of the chromosomes (28). Consequently, probe made by inter-Alu-PCR from a human chromosome DNA will bind preferentially, not only to the human chromosome from which it was derived, but also to the longitudinally-specific regions of that chromosome that are rich in Alus. Therefore, the procedure will not merely paint the human chromosome of interest, but will paint on the chromosome the specific banding pattern unique to it.

Since the centromeric regions of chromosomes are rich in the highly repetitive alphoid repeat sequences (which are a different class of repeat sequence than Alu -- 24), they are not amplified by the inter-Alu-PCR. Consequently the centromeric regions of the chromosomes

-25-

are not painted by probe made by this method (e.g.,
Figure 4). Painting probes which include centromeric
regions are often not chromosome specific because those
non-chromosomal specific regions are not readily blocked
5 (due to the *highly* repetitive clustered nature of such
sequences, as opposed to the *middle* repetitive status of
the interspersed Alu repeats) or eliminated from the *in-*
situ hybridization reaction (12,13). Eliminating such
regions in the design of our probes enhances their
10 chromosomal specificity.

One of the special advantages of making *in-situ*
hybridization probe for specific chromosome regions by
inter-Alu-PCR from hybrid cells containing only those
15 regions of the human genome is the flexibility provided
by being able to prepare probe that identifies only
chromosomal arms, regions or sub-regions. Such probe can
readily identify chromosomal abnormalities involving only
a single chromosome (inversions or deletions) as
20 indicated in Example 8.

In addition chromosome region-specific probes make
it possible to identify and resolve those regions in an
interphase nucleus (the compartment of the cell cycle in
25 which upwards of 90% of the cells are located). In
interphase, the chromosomes are not visible because they
are not condensed. It is a well recognized phenomenon
that *in-situ* hybridization probe for a specific
chromosome will identify the domain that chromosome
30 occupies in an interphase nucleus (8,9). Since
chromosomes are "unwound" in that phase, entire
chromosomes present very large, unresolved domains of
hybridization. By being able to make probe from only a
small fraction of a chromosome, the domain visualized
35 becomes smaller and better resolved. The identification
of chromosomes and chromosomal abnormalities in such

-26-

nuclei then becomes possible. The demonstration of such resolution using such probes and indication of their usefulness in observing intra-chromosomal abnormalities in interphase nuclei is cited in Example 9.

5

However, the identification of translocations in interphase nuclei by using combinations of two such probes tagged so that they will fluoresce in two different colors and which are derived from small regions of the affected chromosomes flanking the translocation breakpoints will be the major application of inter-Alu-PCR probes to interphase nuclei. The translocation will be observable as a combination of the two colors in those nuclei. Since these chromosome regions, from which probes are made by the invented method, are measured in millions of base pairs in length the signal they give will be quite outstanding and observable in every cell nucleus without the need for computer enhancement (as in Example 9). This distinguishes them from present methodology in which human cosmid clones flanking the translocation breakpoint sites are labeled with different color probes and used to observe the translocations in interphase nuclei (23). Since the cosmid clones are only tens of thousands of base pairs in length, the signal is weak and not reliably visualized in every interphase.

This ability to reliably determine if the translocation chromosome is present in interphase nuclei will be of major importance in the management of minimal residual disease during cancer chemotherapy. In such cases one wishes to determine the low frequency of cells in the bone marrow of a leukemia patient that had, or is undergoing, treatment. Since that frequency is low (perhaps 1/1000 or 1/10,000) it is impractical to make the determination from only the few cells in metaphase that one recovers from such preparations. It is also

-27-

likely that the cells in metaphase might be a disproportionate representation of a single cell type and not representative of the population of cells as a whole. The ability to see the translocation in the interphase
5 cell will not only be an excellent monitor for the effectiveness of treatment but also be an extremely useful research tool in determining the etiology of the disease.

10 While the procedure was designed for preparing *in-situ* hybridization probe by amplifying human chromosomal regions isolated in hybrid cells, the inter-Alu-PCR method will also be effective in providing probe from
15 chromosome regions isolated by other methods -- flow-sorted human chromosomes (8,9); chromosome regions cloned into large insert microbial vectors such as yeast artificial chromosomes (YACs -- 29); or chromosome regions scraped by microdissection from a microscope slide (30). In these cases it will not be necessary to
20 increase the annealing temperature for the PCR reaction since there is no danger of amplifying contaminating (non-human) DNA.

While the innovative procedure for blocking repeat
25 sequence DNA from participating in the *in-situ* hybridization reaction is effective, the present invention also includes a method to completely remove repeat sequences. Therefore, the hybridization reaction may be conducted with pure unique sequence specific to
30 the chromosomal region of interest. As indicated above, there are two sources of repeat sequence in the inter-Alu-PCR probes that require elimination. The first source is present because of the fact that primers Alu-1 and Alu-2 are not located at the very ends of Alu
35 segments (the very ends, although still Alu, are not sufficiently conserved to be able to identify a useful

-28-

consensus sequence for use as primer). Therefore all amplification products will have at least 62 to 120 bp of terminal Alu sequences in them (depending upon whether a segment was the amplification product of priming from Alu-1 to Alu-1, Alu-1 to Alu-2, or Alu-2 to Alu-2). The second species of repeat sequences present in probe are those repeat sequences which happen to be located between Alu sequences which, along with the desired unique sequence, get amplified as a result of the inter-Alu-PCR. In order to more effectively block the Alu ends, Alu-1 and Alu-2 primers are to be used in PCR reactions with primers that recognize the arms of a plasmid containing the complete Alu sequence (BLUR2 -- 31). This will amplify those same terminal Alu sequences that were amplified by the inter-Alu PCR. Those amplified terminal Alu sequences, along with low-Cot DNA (described above and which represents additional Alu segments and other species of repeat sequences present in the inter-Alu-PCR product), may be covalently linked to diazobenzyloxymethyl cellulose (32,33) and hybridized with the labeled probe (34,35) following nick-translation and biotinylation. Biotinylated fragments containing the repeat sequences will hybridize to those sequences bound to the cellulose and may be centrifuged out. Remaining biotinylated probe (containing only chromosome-specific unique sequences) will then be directly hybridized to metaphase spreads to detect the specific human chromosome regions of interest. An economical feature associated with the use of the diazonium cellulose with the chemically bonded repeat sequences is that the biotinylated fragments, attached to them after a reaction cleaning up the inter-Alu PCR reaction, can be removed by simply reheating the complex. This regenerates the diazonium cellulose with its bound repeat sequences for reuse in cleaning up more inter-Alu-PCR product.

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While this invention has been described in terms of providing probe for specific regions of the human genome so that those areas can be visualized by *in-situ* hybridization in order to detect any possible alteration of those regions in cancer cells or cells of a developing embryo, such probe may also be used to isolate specific human genes located in those specific chromosomal regions. This is done by using probe made by any of the options above to screen tissue specific cDNA libraries. cDNA libraries are recombinant DNA molecules packaged usually into phage hosts in which the human DNA represents the human genes transcribed in those particular tissues. This technique will be extremely useful when genetic studies reveal, for instance, that the gene for Alzheimer's disease is located on a specific region of human chromosome 19. Then by making inter-Alu PCR probe for that specific region of human chromosome 19, brain cDNA libraries could be screened to isolate the human genes from that region for identification of candidate genes for the disease.

Materials and Methods

Preparation of DNA template from hybrid cells for inter-Alu PCR

DNA template from hybrid cells may be prepared by several methods, two of which are described. The first method is most preferred in the practice of the invention.

1. DNA in Solution. DNA was isolated in traditional fashion by methods well known to those skilled in the art and are as described (36).

35

2. DNA in Agarose Plugs. Preparation of genomic DNA in

-30-

agarose plugs was performed according to van Ommen and Verkerk (37). For PCR, the plugs were washed in 10 mM Tris-HCl pH7.5 for four changes at room temperature, 15 minutes each, then melted at 65°C for 5 minutes. 10 mM
5 Tris-HCl pH7.5 was added to a final volume of 500 µl. The size of the DNA was reduced by vortexing for 30 sec. before storage at 4°C. 10 µl was used for each PCR. This method also gave satisfactory results but was less preferred to method one above.

10

PCR protocol

The consensus inter-Alu-PCR primers designed were:

15

Alu-1: GGATTACAGGYRTGAGCCA, and

Alu-2: RCCAYTGCACTCCAGCCTG

where Y is either pyrimidine (T or C) and R is either
20 purine (A or G). Therefore the Alu-1 primer is actually a cocktail composed of the 4 possible Alu-1 molecules (T at bp 11 and A at bp 12; T at bp 11 and G at bp 12; C at bp 11 and A at bp 12; and C at bp 11 and G at bp 12). Similarly Alu-2 is a cocktail of the 4 possible Alu-2
25 molecules with all possible combinations of alternative purines and pyrimidines at positions 1 and 5 respectively.

In a final volume of 50 µl, was added 100 ng genomic
30 DNA in solution or 10 µl of melted agarose plug, 5 µl of 10 x PCR buffer (100 mM Tris-HCL, pH 8.3, 500 mM KCL, 15 mM MgCl₂ and 0.01% gelatin), 0.2 mM dNTP, 50 pmoles of each of the two primers, and 1.25 units (0.25 µl of 5 unit/µl) Taq polymerase from Perkin-Elmer Cetus. The PCR
35 reaction was conducted using a thermal cycler from Precision Scientific, Model GTC-1. After an initial 5

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min incubation at 94°C, samples were subjected to 25 cycles as follows: 94°C 1 min, 65°C 1 min (a higher was used instead of the standard annealing temperature of 55°C in order to amplify only human sequences in the presence of other mammalian Alu sequences, such as in a hybrid cell -- for PCR from YACs, phage or any source where human Alus are the only ones present the standard annealing temperature would be preferable), and 72°C 3 min. Finally an elongation of 10 min at 72°C was conducted. One-tenth aliquots of each sample were run on gel. Primers and free nucleotides were removed with Centricon-100 (Amicon).

Evaluation of PCR product on agarose gels

500 ng of PCR product was loaded on a 0.8% agarose gel (7 cm x 7 cm) and run at 80 - 100 volts for 15 - 45 min. Ethidium bromide was incorporated into the gel and the running buffer by adding 5 µl ethidium bromide to 500 ml running buffer. Running buffer was:

Tris, 121.16 g
boric acid, 61.86 g
EDTA, 7.46 g
up to 4 l with water, pH brought to 8.3

Gels were photographed by Polaroid camera at f4.5 for 2 sec using #667 film while being transilluminated by UV light.

Nick translation to label the inter-Alu PCR product with biotin for use as probe

Reaction components were from BRL nick translation kit, Cat. No. 8160SB. (BRL, Gaithersburg, MD).

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x μ l PCR product (1 μ g DNA)
5 μ l A1
2.5 μ l 0.4mM Bio-7-dATP
y μ l H₂O
5 45 μ l
add 5 μ l solution C (DNA pol.1 and DNase)
50 μ l

Product was mixed, spun down, and then incubated at
10 16°C for 90 min. 5 μ l stop buffer was added and the
mixture then run through a Worthington Sephadex column to
remove free nucleotides. A check for DNA was made using
100 ng on minigel. The size of inter-Alu PCR product
ranged from 0.1->4kb and the size of the nick translation
15 product ranged from 100-400 bp.

Slide Preparation

1. Freshly fixed (acetic acid-methanol) slides of cells
20 in log phase growth were prepared by standard methods.

2. RNase treatment. 100 ug/ml, 1 hr., moist chamber,
37°C, rinse in 4-6 changes in 2xSSC, dehydrate through
ethanols.

25

3. Denature DNA. Immerse in 70% formamide (in 2x SSC)
at 71°C for 5 min. Dehydrate in ethanol on ice. Dry.

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Probe Cocktail for In-Situ Hybridization -- Preparation and Application

In an Eppendorf tube was added 400 ng biotinylated
5 probe DNA, 20 ug of Cot 10 human DNA (see next paragraph)
and 5 ug of sonicated salmon sperm DNA. The DNA was
dried down, and resuspended in 5 μ l H₂O before adding 12.5
 μ l formamide, 2.5 μ l 10xSSC, and 5 μ l 50% dextran
sulphate. Probe was denatured at 75°C for 5 minutes,
10 pre-anneal at 37°C for 20 minutes. The probe was applied
on one slide, covered with a 22 x 30mm coverslip and
Incubated at 37°C overnight in moist chamber.

Washes were conducted at 41°C: 3 changes in 50%
15 formamide in 2 x SSC for 3 min. each, then 5 changes in 2
x SSC for 2 min each stirring vigorously. Storage was in
the dark in BT buffer, pH 8.0.

Cot 10 human DNA was prepared from placental DNA,
20 purchased from Sigma. It was dissolved in TE and
sonicated to an average size of 500 bp. Then 20 mg of
DNA was denatured and incubated at 65°C in 2 ml 5 x SSC
until Cot10. The DNA solution was quickly chilled on ice
and 10 fold excess of nuclease S1 buffer (33 mM NaAc,
25 pH4.5 and 1 mM ZnSO₄) and 800 units of nuclease S1 were
added. After 2 hours incubation was carried out at 37°C
to digest single-stranded DNA before removing nuclease S1
with proteinase K digestion. Finally the DNA was
purified with phenol/chloroform extraction and ethanol
30 precipitation.

Fluorescent Staining

The following steps were utilized in fluorescent
35 staining procedures.

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1. Drain and blot off BT buffer and add 55 μ l 5% BSA in BT buffer and cover with plastic cover slip 5 min at room temp.
- 5 2. Peel off cover slip, drain fluid and put on 55 μ l fluorescein avidin solution (2.5 μ l fluorescein avidin (FA) and 247.5 μ l 5% BSA in BT buffer). (That comes to 0.55 μ l FA.) Cover with plastic cover slip, in moist chamber 37°C for 1 hr.
- 10 3. Wash in BT buffer at 40°C 3x. Store as before in dark in BT buffer at room temp.
- 15 4. Blot and apply 55 μ l 5% goat serum in BT buffer and cover with plastic cover slips for 5 min. at room temp. Peel off cover slip, drain fluid and add 55 μ l of biotinylated anti-avidin solution (12.5 μ l biotinylated anti-avidin plus 237.5 μ l 5% goat serum in BT buffer and recover with plastic cover slip). Incubate in moist
20 chamber at 37°C for 1 hr. and wash as in "3".
5. Add another layer of fluorescein avidin as in "2" above.
- 25 6. Repeat "3", "4", and "5" to put on a 3rd layer.
7. Wash and store as in "3".
8. Counterstain for 90 sec. in 2 μ l/ml propidium iodide
30 in deionized water. Mount with antifade solution (38).

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Microscopy

Photomicrographs were obtained using a Zeiss Epi-illumination Photoscope with filter combination 48 77 09
5 and photographed on Kodak Ektachrome 160 with exposure times between 30 and 50 seconds.

The following examples are intended to illustrate the practice of the present invention and are not
10 intended to be limiting.

Example 1

15

Selective Amplification of Human DNA with Alu-1 and Alu-2.

The primers were tested for their specificity for
20 amplification of human DNA. At standard conditions for PCR (which include annealing of primer to template DNA at 55°C) both primer sets Alu-1 and Alu-2, either alone or in combination, amplified rodent as well as human DNA. This is demonstrated in Panel A of Figure 1. The
25 products of the PCR reaction conducted on the DNA from human HeLa cells (human) and CHO cells (Chinese hamster) were visualized after electrophoretic separation on an agarose gel. A smear of product (indicating DNA in a wide range of molecular weights (from 100 bp to 20,000
30 bp) was produced in CHO as well as human DNA. However, raising the annealing temperature to 65°C successfully amplified human but not CHO DNA. This is indicated in Panel B of Figure 1 where a wide size range of DNA was produced from human DNA but none from CHO.

35

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Example 2

The efficiency of the Alu-1 and Alu-2 primer sets were compared with Alu-PCR primers TC-65 or 559 previously published (19) and used to generate *in-situ* hybridization probe (20). This was done by using a series of cosmids containing 20,000 bp to 40,000 bp of human DNA as substrate for PCR reactions and separating the products on agarose gels. The more efficient primers were expected to prime more frequently and therefore produce a wider spectrum of DNA bands from the cosmids. As shown in Figure 2, Alu-1 and Alu-2 primers (O) produced a significantly greater number of bands of human genomic DNA than were produced using the previously published primer (N) from each cosmid.

Example 3PCR Production of Human Probe from Hybrid Cells

Cell line 5HL9-4 is a human x CHO somatic cell hybrid containing chromosome 19 as its only human genomic element (39). This is demonstrated in Figure 3 where using total human DNA as *in-situ* hybridization probe on the cells revealed only a single human chromosomal element as brightly fluorescing. Conducting PCR with Alu-1 and Alu-2 as primers, at the annealing temperature (65°C) specific for human DNA, produced a smear of human DNA from 100 bp to 20,000 bp in length. Therefore, using the specific hybrids and conditions for PCR, human specific probe for *in-situ* hybridization could be prepared from DNA of a hybrid cell in which the human DNA represented less than 5% of the total in the cell.

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Example 4Painting of a Specific Human Chromosome

5 Inter-Alu-PCR DNA from 5HL9-4 was used for *in-situ*
hybridization on a human metaphase cell after
biotinylating the PCR product, blocking non-chromosome
specific repeat sequences with low Cot DNA and
visualizing the sites of hybridization with the avidin-
10 fluorescence reaction. The results, shown in Figure 4,
clearly indicated human lymphocyte metaphases in which
the only brightly fluorescing human genetic elements were
the two human number 19 chromosomes. Significant
fluorescence was present on only the two human number 19
15 chromosomes while fluorescence on other chromosomes was
effectively quenched. Note the absence of fluorescence
at the centromere of this and all other chromosome
specific probe produced by inter-Alu-PCR. At this level
of contraction, each chromosome arm of chromosome 19
20 appears as one large R-band.

Example 5Painting a Specific Chromosome Arm

25 The procedure described was used to paint only part
of a human chromosome. Figure 5 indicates a hybrid cell
(41XP91-3) containing a fragment of a human chromosome.
41XP91-3 is one of a series of hybrid cells made between
30 human xeroderma pigmentosum cells and the DNA repair
deficient CHO cell line UV41. Hybrids were selected for
the complementation of the missing DNA repair function in
UV41 (selection in mitomycin C). Biochemical and
molecular analysis of the hybrids indicated that the
35 human repair gene complementing the lost function in UV41
must be on human chromosome 16 since genetic markers

-38-

identifying the 16 were present in every hybrid while other human chromosomes were only randomly present (40). Furthermore, the analysis indicated that 41XP91-3 had markers only from the p-arm of chromosome 16 and
5 contained no markers representing any other region of the genome. That, plus the cytogenetic analysis exemplified in Figure 5, allowed us to conclude that the hybrid cell line contained only a portion of human chromosome 16 as its only human content. Probe was prepared and applied
10 to human metaphases. As seen in Figure 6 only the p-arm of chromosome 16 lights up.

Example 6

15 Painting a Specific Sub-Region or Band of a Chromosome

Probe was prepared from a hybrid (2F5) containing only a small fragment of human genetic material attached to a CHO chromosome (Figure 7). 2F5 was derived from a
20 human x CHO hybrid (20XP3542-1-4) which was shown to have approximately 25 Mb of human DNA derived largely from a portion of human chromosomes 17 (41) and a small region from the q-arm (q13.3) of chromosome 19 in the vicinity of the DNA repair gene ERCC1 (42). 2F5 was prepared from
25 20XP3542-1-4 by X-irradiation of the former and hybridization back to the CHO cell line that was its original rodent parent (UV20). Radiation hybrids were selected for ERCC1 by growth in mitomycin C (43). Analysis of molecular markers retained in 2F5 indicated
30 that it had lost all human DNA except the region of ERCC1 on chromosome 19 and that the total amount of human DNA in the hybrid was between 1 Mb and 2 Mb (D. Shaw, Cardiff, Wales, U.K., personal communication). The in-situ hybridization probe made by inter-Alu-PCR from 2F5
35 detected only band p13.3 on every human chromosome 17 in all human metaphases seen (over 50; e.g., Figure 8).

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Example 7Painting Specific Chromosomes With a Banding Pattern

5 Probe was prepared from a hybrid containing only a
single human genetic element which molecular analysis
indicated to be a human chromosome number 7. When probe
was made from this hybrid and used for *in-situ*
hybridization on human metaphases (Figures 9 and 10), the
10 two chromosome 7s lit up in an R-banding pattern.

Probe was made from the DNA of a hybrid known to
contain only chromosome 5 (obtained from L. Nagaragian,
Department of Hematology, University of Texas M.D.
15 Anderson Cancer Center, Houston, TX). As shown in figure
11, chromosome 5 is the only painted element in a human
metaphase spread. The distinctive R-band pattern is
identifiable in this somewhat overly contracted state.

20 Inter-Alu-PCR probe made from a hybrid containing
only human chromosome 17 (obtained from David Ledbetter,
Baylor College of Medicine, Houston, TX) specifically lit
up chromosome 17 in human metaphase spreads (Figure 12).
The chromosome is small and highly contracted in this
25 metaphase. As seen in Figure 13, the R-band patterns are
resolved and the chromosome 17s are observed in
metaphases where chromosomes are not well spread out.

Probe made from a hybrid containing human chromosome
30 3 as the only human element was characterized and
obtained from Dr. Susan Naylor (University of Texas
Health Science Center at San Antonio, TX). When applied
to a human metaphase spread this probe specifically
hybridized to human chromosome 3 in an R-banded pattern
35 (Figure 14) supporting the hypothesis constructed after
observing the results with the chromosome 7 probe.

-40-

In addition to the human chromosomes presented in the examples, the method has been used to paint R-band patterns on chromosomes 9 and 16.

5

Example 8Detection of Chromosomal Abnormalities

From the examples shown, it is clear that
10 translocations between one chromosome and another will be obvious and easily detected using a chromosome specific probe. For instance, where a translocation existed between one of the chromosome 19s and one of the 17s, probe from the 19 would readily detect a single, entirely
15 fluorescing chromosome (the 19 not involved in the translocation) and two other chromosomes with only portions fluorescing (the 19 with a piece of the 17 and the 17 with a piece of the 19). By the same token, it will be even easier to identify monosomies (a single
20 fluorescing chromosome instead of two in the cell), and trisomies (three fluorescing chromosomes). A special advantage in working with probes for only a portion of a human chromosome or which light up a banding pattern on a chromosome is that it will enable the identification of
25 inversions and also the specific sites of deletions and translocations.

Detection of an Inversion

30 A chromosome 16 p-arm specific probe as described in Example 4 above was used to identify the inversion 16 chromosome associated with acute nonlymphocytic leukemia. Since the inversion breakpoint on the p-arm cuts right through the painted region of the chromosome, the
35 inversion chromosome was readily identified as a chromosome having fluorescence at the end of the p-arm

-41-

and also in the middle of the q-arm (Figure 15).

Detection of the Site on a Deletion

5 Chromosome 7 specific probe which lit up a banding
pattern on chromosome 7 was used to identify the location
of a deletion on the 7 associated with an AML cell line
KB-1 (provided by Jan Liang, Division of Laboratory
10 Medicine, University of Texas M.D. Anderson Cancer
Center, Houston, TX). When applied to the cell, the
number 7 chromosomes were immediately identifiable with
the normal 7 quite distinct from the deleted 7. The site
of the deletion was readily detected as just distal to
the most proximal bright band on the q-arm as shown in
15 Figure 16.

Example 9

Identification of Chromosome Regions in Interphase Nuclei

20

Chromosome Regions in Normal Cell Interphases

In the interphase nuclei, chromosomes are "unwound"
or drawn out to the extent that they are no longer
25 individually identifiable. Using the probes that are
specific for an entire human chromosome, it is possible
to identify but difficult to resolve the "domains" within
an interphase nucleus in which the particular chromosomes
exist. However, by using probe made from a hybrid
30 containing a highly limited region of a particular
chromosome, such domains are clear, bright and highly
resolved. This is demonstrated by using probe made from
hybrid 2F5 (described in Example 6 above) which contains
only 1 - 2 Mb of human DNA from band q13.3 of human
35 chromosome 19. As seen in Figure 20, such probe gives
clear resolution of the chromosomal region in interphase

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nuclei.

Chromosomal Abnormalities in Interphases

5 Probe for any normal chromosome will identify two domains in an interphase nucleus. Where there has been a translocation involving a chromosome three, poorly resolved domains may be visible. Three domains were expected from the inversion 16 chromosome described in
10 Example 8a above. Figure 19 shows that by using probe for only part of the chromosome in such an interphase nucleus, the normal 16 appeared as a single elongated domain of fluorescence representing the uninvolved 16p arm. Also seen was a second elongated domain divided by
15 a zone of non-fluorescence as expected for the chromosome bearing the inversion.

PROPHETIC EXAMPLE 10

20 The present example outlines the procedure contemplated by the Applicants to be useful for the isolation of genes that have been localized to a known region of the genome.

25 Probe is made from a specific region of the human genome where the gene of interest is known to be localized (via classical genetic mapping studies in families where that gene is known to be segregating, for example). A gene demonstrated to map into the band q
30 13.3 of chromosome 19, for example, is screened from cDNA libraries made from genes expressed in the human brain using inter-Alu-PCR probe made from the hybrid 2F5 (which retains band 19q13.3 as its only human genetic material). This will result in isolation of human brain
35 genes from the genome region in which the desired gene is known to exist, providing candidate genes for the gene of

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interest. Isolation of such a gene would be viewed as important in the characterization, diagnosis, counselling and treatment of any disease associated with that gene.

5 Figure 20 is a schematic illustration of the invention. In order to illustrate a particular example of how the primer sets work, a human Alu repeat sequence is shown in Figure 20A. Figure 20B illustrates how nonrepeat DNA regions are selectively amplified.

10 Although the figure is illustrated using human Alu repeat segment, the same general scheme applies to the preparation of chromosomal specific DNA probes using other species specific repeat sequences.

15 The present invention has been described in terms of particular embodiments found by the inventors to comprise preferred modes of practice of the invention. It will be appreciated by those of skill in the art that in light of the present disclosure numerous modifications and changes

20 can be made in the particular embodiments exemplified without departing from the intended scope of the invention. For example, primers and primer sets to repeat sequences other than Alu could be designed using the principles set forth without affecting the intended

25 scope of the invention. All such modifications are intended to be included within the scope of the claims.

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CLAIMS:

1. A method for the production of chromosome specific DNA probes essentially free of species specific repeat DNA sequences, comprising the steps:
 - identifying a desired chromosome or portion thereof from a species to which chromosome specific probes are desired;
 - obtaining DNA from a source in which the desired chromosome or portion thereof has been isolated from the species total chromosomal material;
 - preparing a first and a second primer set, each primer set capable of hybridizing to a region within a species specific repeat DNA segment wherein the first primer set binds at or near the repeat segment 5' terminus of the chromosome DNA and facilitates DNA replication in the direction of that terminus and the second primer set binds at or near the repeat segment 3' terminus of the chromosome DNA and facilitates DNA replication in the direction of that terminus;
 - annealing the primer sets with the DNA obtained from the source in which the desired chromosome or portion thereof has been isolated from the species total chromosomal material; and
 - amplifying the DNA from inter-repeat sequence regions of the desired chromosome or portion thereof to produce DNA probes essentially free of species specific repeat sequences.

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2. The method of claim 1 wherein each primer set comprises at least one oligonucleotide.
- 5 3. The method of claim 1 wherein the chromosome or portion thereof identified is characterized as having DNA specific for the chromosome or portion of the chromosome.
- 10 4. The method of claim 1 wherein the DNA probes are essentially free of alphoid, LINE, or SINE repeat sequences.
- 15 5. The method of claim 4 wherein the SINE is Alu.
6. The method of claim 1 wherein the chromosome or portion thereof identified is characterized as having species specific Alu segments.
- 20 7. The method of claim 1 wherein the amplifying is by polymerase chain reaction.
- 25 8. The method of claim 1 wherein the chromosome or portion thereof is human.
- 30 9. The method of claim 8 wherein the chromosome portion is a chromosome segment isolated in an interspecific cell hybrid or a yeast artificial chromosome.
- 35 10. The method of claim 1 wherein the first primer set

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binds to at least a portion of a species specific repeat DNA consensus sequence at or near the repeat sequence 5' terminus.

5

11. The method of claim 1 wherein the first primer set binds to a species specific repeat DNA consensus sequence located at or within 50 base pairs of the repeat sequence 5' terminus.

10

12. The method of claim 1 wherein primers comprising the first primer set are characterized as having a reverse complementary sequence to a consensus sequence located within a repeat segment.

15

13. The method of claim 12 wherein the repeat segment is a human Alu segment.

20

14. The method of claim 12 wherein the consensus sequence is located 13-31 base pairs from a human Alu 5' terminus.

25

15. The method of claim 1 wherein primers comprising the second primer set bind to a species specific repeat consensus sequence located at or near the 3' terminus.

30

16. The method of claim 1 wherein primers comprising the second primer set bind to a species specific repeat sequence located at or within 100 base pairs of the repeat sequence 3' terminus.

35

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17. The method of claim 1 wherein primers comprising the second primer set bind with a human Alu consensus segment located 240-258 base pairs from an Alu 3' terminus.

5

18. The method of claim 17 wherein primers comprising the second primer set are characterized as having a sequence identical to the human Alu repeat sequence located from base pair 240-258.

10

19. The method of claim 1 wherein each first and second primer set is characterized as comprising at least one primer, said primer or primers binding with a plurality of species specific repeat segments.

15

20. The method of claim 19 wherein the primer or primers comprising the first and second primer sets are further characterized as binding essentially only to human Alu segments.

20

21. The method of claim 1 wherein obtaining DNA of the desired chromosome isolated from the species total chromosomal material is by flow sorting.

25

22. The method of claim 1 wherein obtaining DNA of the desired chromosome or portion thereof isolated from the species total chromosomal material is by microdissection.

30

23. The method of claim 1 wherein the source of the desired desired chromosome or portion thereof is interspecific somatic cell hybrids.

35

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24. The method of claim 1 wherein DNA obtained from interspecific somatic cell hybrids is annealed to primers at a temperature between about 60°C and 80°C.

5

25. The method of claim 23 wherein the annealing temperature is 65°C.

10 26. The method of claim 23 wherein the interspecific somatic cell hybrid is hamster x human.

15 27. The method of claim 1 wherein the source of the desired chromosome portion is artificial yeast chromosomes.

20 28. A method of producing DNA probes specific for a human chromosome or portion thereof, comprising the steps:

25 obtaining DNA from a source in which the desired human chromosome or portion thereof has been isolated from other human chromosomal material;

30 preparing a first and second primer set, each primer set capable of hybridizing to a region within a human specific repeat DNA segment wherein the first primer set binds at or near the repeat segment 5' terminus of the desired human chromosome DNA and facilitates DNA replication in the direction of that terminus and the second primer set binds at or near the repeat segment 3' terminus of the desired human chromosome DNA and facilitates DNA replication

35

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in the direction of that terminus;

annealing the primer sets with the DNA obtained from
the source in which the desired chromosome or
5 portion thereof has been isolated from the
human total chromosomal material; and

amplifying the DNA from inter-repeat sequence
regions of the desired chromosome or portion
10 thereof to produce DNA probes essentially free
of human specific repeat sequences.

29. The method of claim 28 wherein each primer set
15 comprises at least one oligonucleotide.

30. The method of claim 28 wherein the first set of
primers binds to a consensus sequence within a human Alu
20 repeat segment.

31. The method of claim 30 wherein the consensus
sequence is located within 50 base pairs of the human Alu
25 repeat segment 5' terminus.

32. The method of claim 30 wherein the first set of
primers binds to a sequence located between base pairs
30 13-31 of a 5' end of a human Alu repeat consensus
segment.

33. The method of claim 28 wherein the primers
35 comprising the first set of primers differ in one or more
base positions whereby said primers bind to essentially

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all sequences located between base pairs 13-31 of human Alu repeat segments.

- 5 34. The method of claim 33 wherein said primers comprising the following base sequence:

GGATTACAGG YRTGAGCCA

- 10 where Y is T or C and R is A or G producing four primers in the set.

- 15 35. The method of claim 33 wherein the first set of primers comprises approximately equal amounts of four primers.

- 20 36. The method of claim 28 wherein the second set of primers binds to a consensus sequence within a human Alu repeat segment.

- 25 37. The method of claim 36 wherein the consensus sequence is located within 100 base pairs of the human Alu repeat segment 3' terminus.

- 30 38. The method of claim 28 wherein the primers comprising the second set of primers differ in one or more base positions whereby said primers bind to essentially all sequences located between base pairs 240-258 of human Alu repeat segments.

- 35 39. The method of claim 38 wherein said primers have the

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following base sequence:

RCCAYTGCAC TCCAGCCTG

5 where Y is T or C and R is A or G producing four primers
in the set.

40. The method of claim 38 wherein the second set of
10 primers comprises equal amount of four primers.

41. A method for painting a human chromosome or portion
thereof in metaphase spreads or interphase nuclei,
15 comprising the steps:

preparing human cell sample in which a particular
human chromosome or portion thereof is to be
detected;

20

preparing a DNA probe by the method of claim 28;

labeling the DNA probe;

25

hybridizing the labeled DNA probe with the cell
sample;

30

detecting the human chromosome or portion thereof in
metaphase spreads or interphase nuclei
hybridized with the DNA probe.

42. The method of claim 41 further comprising blocking
35 or removing non-chromosome specific repeat sequences that
may be present in the DNA probe.

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43. The method of claim 41 wherein R-banding patterns are painted onto specific human chromosomes in metaphase spread.

5

44. The method of claim 41 wherein a human chromosome or portion thereof comprises at least 500 chromosome specific DNA base pairs.

10

45. The method of claim 41 wherein the particular human chromosome or portion thereof detected is 100-2000 kb.

15

46. The method of claim 41 wherein the labeling is with biotin, digoxigenin, dinitrophenol, aminoacetylfluorene, or conjugated mercury.

20

47. The method of claim 41 wherein the detecting is by fluorescence microscopy following conjugation with an antigenic fluorochrome.

25

48. The method of claim 47 wherein the antigenic fluorochrome is avidin-fluorescein.

30

49. The method of claim 42 wherein the blocking comprises binding non chromosome specific regions of the DNA probe with middle and highly repetitive DNA isolated from total human DNA.

35

50. The method of claim 49 wherein the middle and highly repetitive DNA has a C₀t value of 10.

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51. The method of claim 42 wherein the removing comprises the following steps:

5 obtaining a plasmid containing a complete human Alu repeat segment;

10 preparing a first plasmid arm primer from a known sequence of the plasmid arm adjacent to the 5' terminus of the Alu insert, wherein the primer will direct DNA synthesis toward an Alu-1 primer, said Alu-1 primer having a base sequence reverse complementary to a consensus sequence near the 5' end of the Alu repeat segment and directing DNA synthesis toward the
15 plasmid arm primer;

20 preparing a second plasmid arm primer from a known sequence of the plasmid arm adjacent to the 3' terminus of the Alu insert, wherein the primer will direct the DNA synthesis toward an Alu-2 primer, said Alu-2 primer having a base sequence identical to a consensus sequence near the 3' end of the Alu repeat segment and directing DNA synthesis toward the plasmid arm
25 primer;

30 polymerase chain reaction amplifying both Alu terminal regions located between each plasmid arm and the Alu consensus sequences binding to Alu-1 or Alu-2 primer;

adding human C₀t 10 DNA;

35 linking the amplified Alu terminal regions and human C₀t 10 DNA to a carrier;

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hybridizing the carrier-linked Alu terminal regions
and C₀t 10 DNA with the labeled probe; and

5 separating carrier with bound non-chromosomal
specific repeat sequences from labeled probe.

52. The method of claim 51 wherein the plasmid is BLUR2
10 plasmid DNA.

53. The method of claim 51 wherein the carrier is
15 diazotized Sephacroyl.

54. The method of claim 51 wherein the carrier is
diazobenzoxymethyl cellulose covalently linked to non-
20 chromosomal specific repeat DNA sequences.

55. The method of claim 1, 28 or 41 wherein the portion
of chromosome is chromosome 16 p-arm, 19 q13.3, 1q, 3q,
25 5q, 7q, 9q or 22 q.

56. The method of claim 1, claim 28 or claim 41 wherein
the desired chromosome is human chromosome 1-22, x-
30 chromosome or y chromosome.

57. A kit useful for preparing probes for inter-Alu-PCR
specific for human chromosomes or parts of human
chromosomes comprising:
35

a transporter being compartmentalized to receive one

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or more container means in close confinement therein;

5 a first container means comprising a set of Alu-1 primers, said primers having the base sequence GGATTACAGG YRTGAGCCA wherein Y is T or C and R is A or G; and

10 a second container means comprising a set of Alu-2 primers, said primers having a base sequence RCCAYTGAC TCCAGCCTG wherein Y is T or C and R is A or G.

15 58. The kit of claim 57 wherein the primer sets are supplied in lyophilized form.

20 59. A kit useful for the *in-situ* painting and banding of human chromosomes or parts of human chromosomes comprising:

25 a transporter being compartmentalized to receive one or more container means in close confinement therein; and

30 a first container means comprising labeled, DNA probes essentially free of species specific repeat sequences capable of binding and painting specific human chromosomes or parts of human chromosomes.

35 60. The kit of claim 59 wherein DNA probes are labeled with biotin.

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61. The kit of claim 59 wherein the DNA probes are supplied in lyophilized form.

5 62. The kit of claim 59 wherein the probes are specific for human chromosomes 1-22, the X and the Y chromosomes.

63. The kit of claim 59 wherein the probes are specific
10 for chromosome parts: 16p, 19 q13.3, 9q, 22q, 5q, 7q, 3p, 1q.

64. A nucleotide primer set comprising the following
15 base sequence:

GGATTACAGG YRTGAGCCA

wherein Y is T or C and R is A or G and wherein the
20 primers are characterized as binding near a 5' Alu terminus in a plurality of human Alu consensus segments.

65. A nucleotide primer set comprising the following
25 base sequence:

RCCAYTGCAC TCCAGCCTG

wherein Y is T or C and R is A or G and wherein the
30 primers are characterized as binding near a 3' Alu terminus in a plurality of human Alu consensus segments.

66. A collection of DNA probes capable of hybridizing to a selected region of a chromosome, said probes
35 characterized as essentially free of species specific repeat segments.

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67. The DNA probes of claim 66 characterized as specific for human chromosomes 1-22, X chromosome, Y chromosome, 16p, 19q13.3, 9q, 22q, 5q, 7q, 3q and 1q.

- 5 68. A method of isolating genes identified as associated with a condition or disease state, comprising preparing a DNA probe of by the method of claim and identifying gene candidates from a cDNA library prepared from a sample suspected of having the gene associated with the
- 10 condition or disease state.

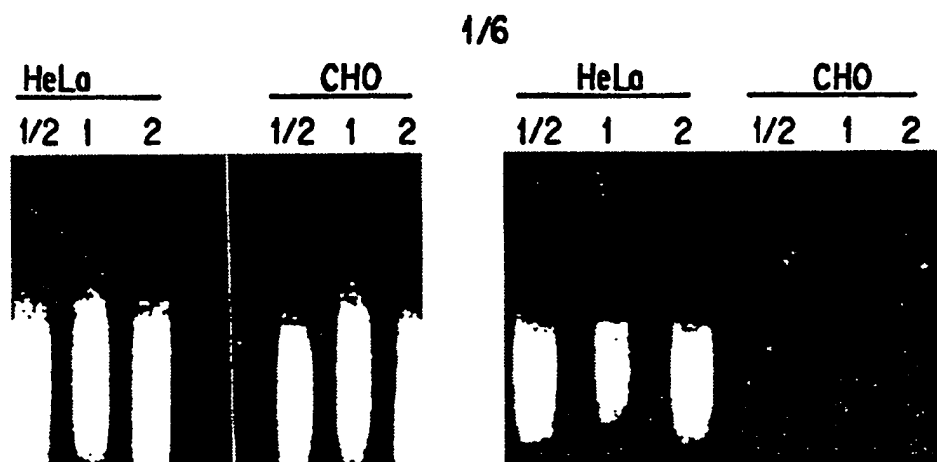


FIG. 1A

FIG. 1B

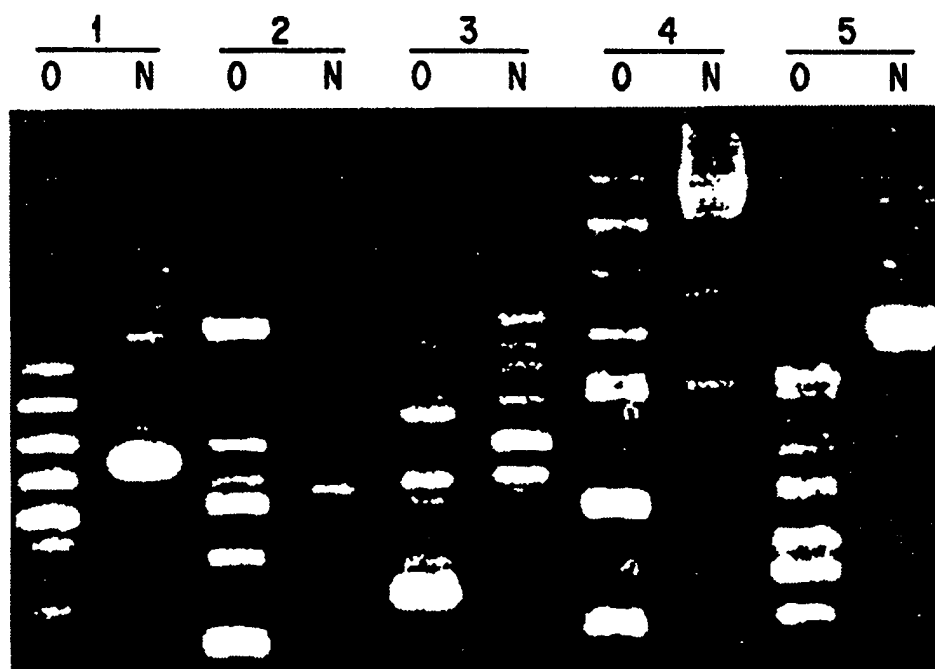


FIG. 2

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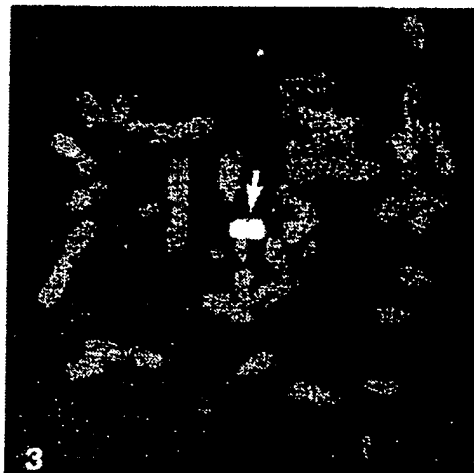


Fig. 3



Fig. 4

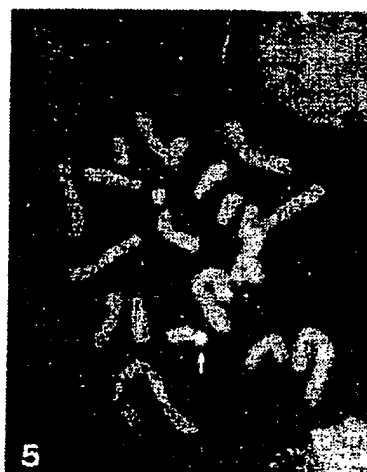


Fig. 5



Fig. 6

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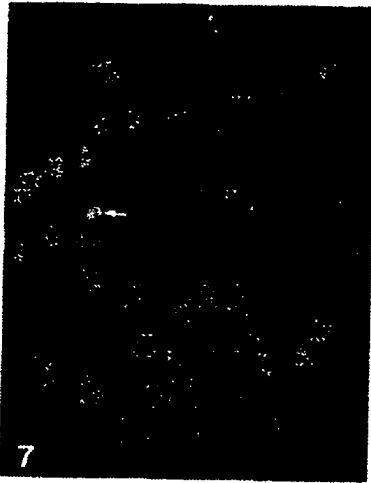


Fig. 7

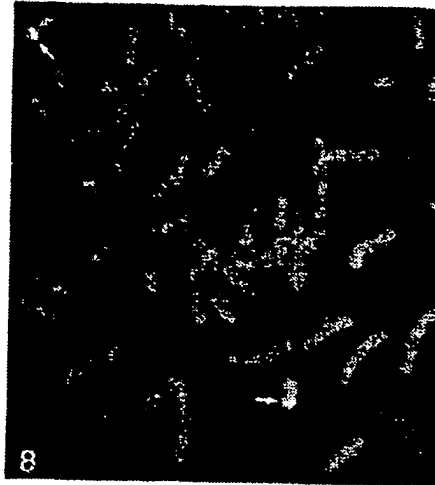


Fig. 8

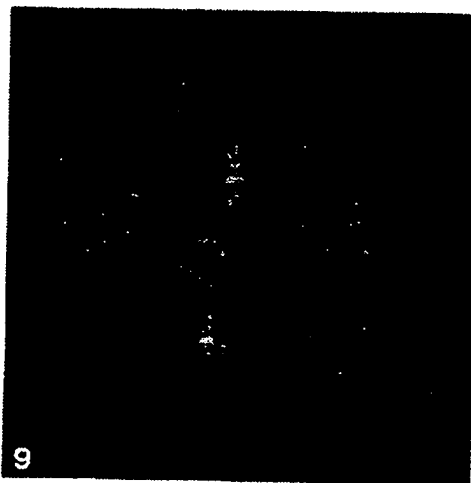


Fig. 9



Fig. 10

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Fig. 11

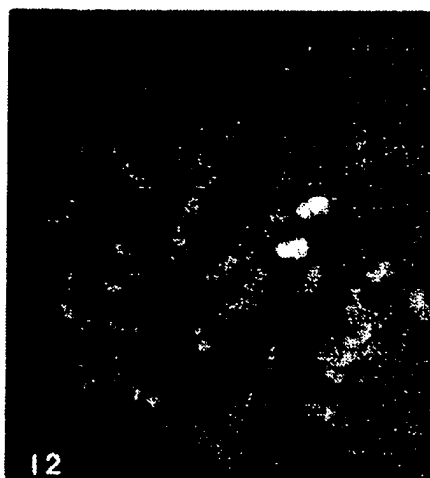


Fig. 12



Fig. 13

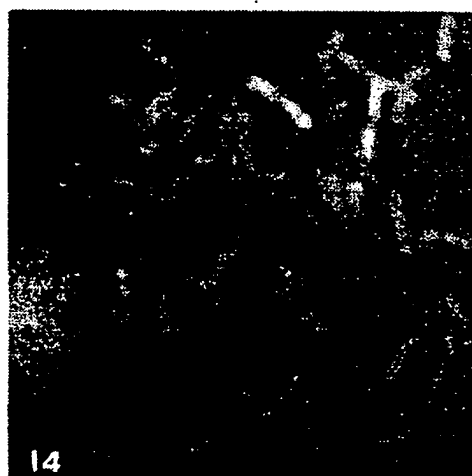


Fig. 14

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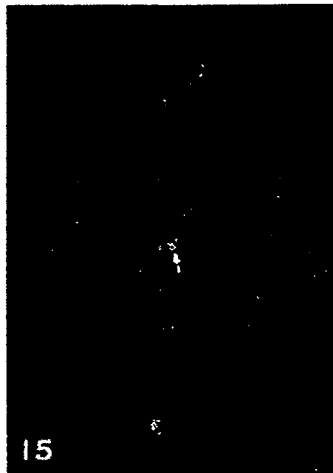


Fig. 15

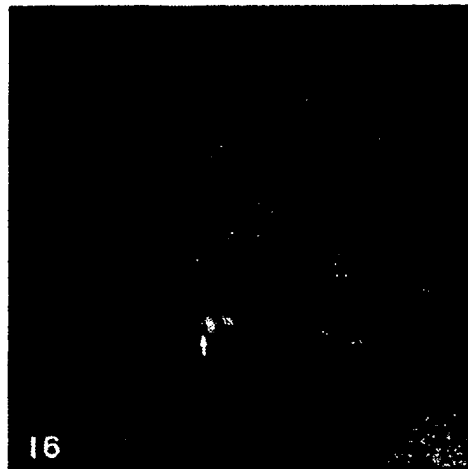


Fig. 16



Fig. 17



Fig. 19

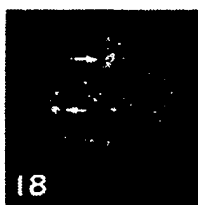


Fig. 18

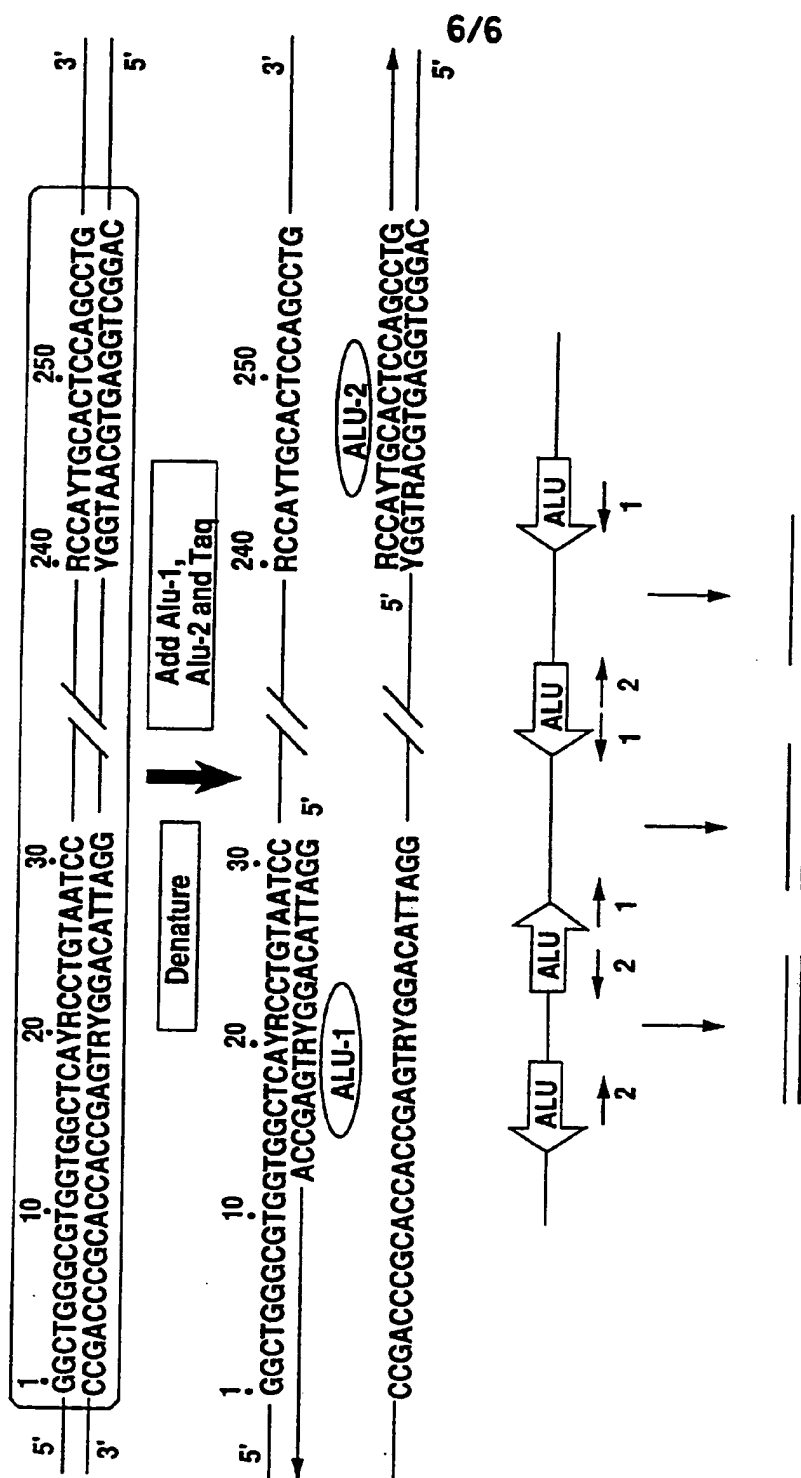
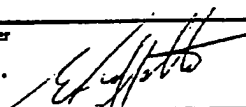


FIG. 20

INTERNATIONAL SEARCH REPORT

PCT/US 91/08739

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/10; C12Q1/68		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C12Q	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	<p>HUMAN GENETICS vol. 86, no. 1, November 1990, BERLIN, DE pages 1 - 6; C. LENGAUER ET AL.: 'Painting of human chromosomes with probes generated from hybrid cell lines by PCR with Alu and L1 primers.' see abstract see page 2, left column, line 45 - right column, line 38</p> <p style="text-align: center;">--- -/-</p>	1,24,41, 66
<p>⁹ Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
14 APRIL 1992	20. 04. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	LUZZATTO E.R. 	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>GENOMICS vol. 7, no. 2, June 1990, NEW YORK, USA pages 257 - 263; F.E. COTTER ET AL.: 'Rapid isolation of human chromosome-specific DNA probes from a somatic cell hybrid' cited in the application see the whole document esp. abstract, page 258, right column, lines 34 to 47 and " Discussion"</p> <p>---</p>	1,28,36,37
X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, September 1989, WASHINGTON US pages 6686 - 6690; D.L. NELSON ET AL.: 'Alu polymerase chain reaction: A method for rapid isolation of human-specific sequences from complex DNA sources' cited in the application see the whole document esp. abstract and fig. 1</p> <p>---</p>	1,56
X	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, September 1990, WASHINGTON US pages 6634 - 6638; P. LICHTER ET AL.: 'Fluorescence in situ hybridization with Alu and L1 polymerase chain reaction probes for rapid characterization of human chromosomes in hybrid cell lines' see the whole document esp. abstract, page 6634, left column, line 23 to right column, line 29 and " Discussion"</p> <p>---</p>	1,26,41
A	<p>WO,A,9 005 789 (YALE UNIVERSITY) 31 May 1990 see page 14, line 6 - page 19, line 23; claims</p> <p>---</p>	1,41,51
P,X	<p>EP,A,0 430 402 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 5 June 1991 see page 21, line 53 - page 22, line 10; figure 7</p> <p>---</p>	1

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9108739
SA 55880**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 14/04/92

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		CN-A-	1046392	24-10-90
		EP-A-	0444115	04-09-91

EP-A-0430402	05-06-91	AU-A-	5898790	06-06-91
		CA-A-	2021489	20-01-91
		JP-A-	3224499	03-10-91
